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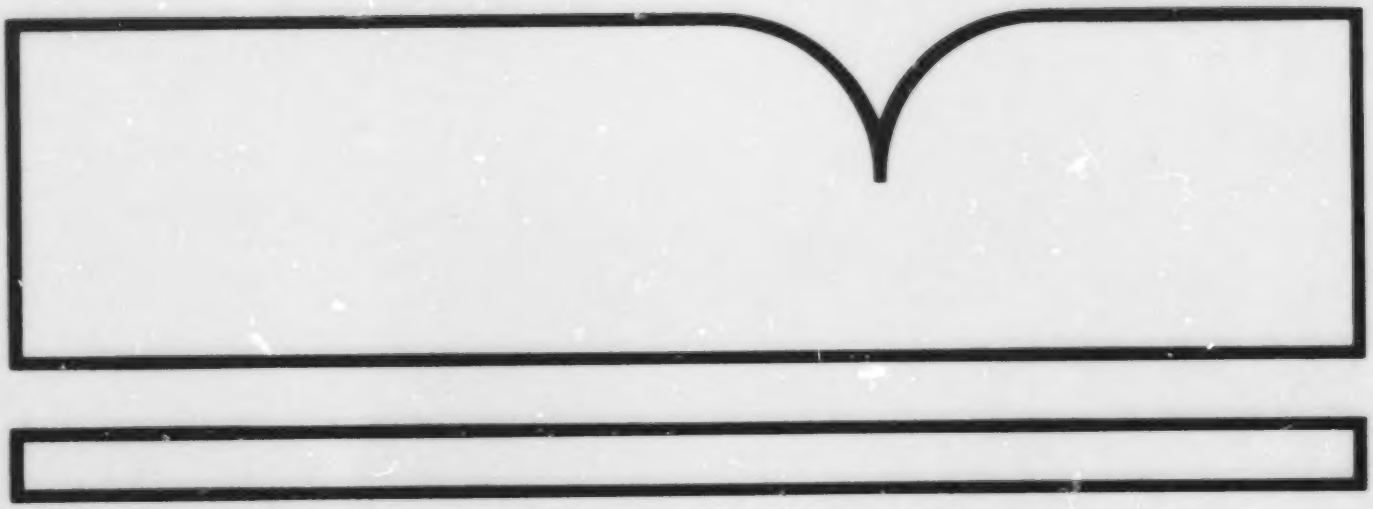

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S9-DEPENDENT ACTIVATION OF 1-NITROPYRENE AND 3-NITROFLUORANTHENE IN BACTERIAL MUTAGENICITY ASSAYS

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16. ABSTRACT

Nitro-substituted polycyclic aromatic hydrocarbons (NO_2PAH) such as 1-nitropyrene (NP) and 3-nitrofluoranthene (3-NFA), both widespread environmental mutagens, generally require activation by bacterial nitroreductases for maximal expression of their mutagenicity in the Ames Salmonella histidine reversion assay; addition of exogenous mammalian drug-metabolishing enzymes (S9 fraction) decreases their activity in this system. In contrast, in an assay of forward mutation (to 8-azaguanine resistance) with Salmonella typhimurium strain TM677, without S9 NFA has relatively low activity (10 to 20 mutants per 10^5 survivors at 5 µg/ml) and NP only marginally doubles the background mutation rate. Addition of S9 protein enhances NP at 50 µg/ml and 80 to 100 x 10^{-5} for NFA at 5 to 10 µg/ml. Therefore generation of active genotoxic intermediates in this forward mutation system may proceed through metabolic pathways other than the previously-defined bacterial nitro-reduction. Previously-identified oxidative metabolites of NP are known to be mutagenic.

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S9-DEPENDENT ACTIVATION OF 1-NITROPYRENE AND 3-NITROFLUORANTHENE IN BACTERIAL MUTAGENICITY ASSAYS.

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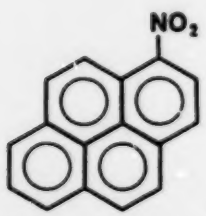
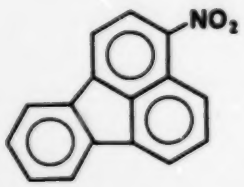
INTRODUCTION

Nitro-substituted polycyclic aromatic hydrocarbons (NO_2PAH) such as 1-nitropyrene (NP) and 3-nitrofluoranthene (NFA; see Fig. 1) have been detected in diesel and other combustion emissions (Nishioka et al., 1982; Gibson, 1982; Schuetzle et al., 1982). Many of these compounds are potent mutagens, and some are animal carcinogens (Hirose et al., 1984; Ohgahki et al., 1982). The mutagenicity of NO_2PAH in the Ames Salmonella typhimurium plate incorporation assay is generally dependent on nitroreductase activity in the bacterial tester strains utilised (Mermelstein et al., 1981). This was shown to be the case for NP (Mermelstein et al., 1981; Ball et al., 1984a). When the bacterial tester strain was deficient in the required nitroreductase enzyme, metabolism by mammalian S9 or microsomal fractions was also able to activate this compound, although to a lesser extent (Kohan and Claxton, 1983; Ball et al, 1984a). Oxidative metabolism of NP both in vivo (Ball et al., 1984b) and in vitro (El-Bayoumy and Hecht, 1983; Ball et al., 1984a) was shown to form phenols and dihydrodiols some of which were themselves mutagenic to Salmonella. Binding to DNA, also an indication of potential for genotoxic damage, was catalysed both by reductive (Messier et al., 1981; Howard et al., 1983) and by NADPH-dependent oxidative metabolism of NP (Ball and Lewtas, 1985). Therefore both oxidative and reductive pathways apppear to be capable of producing genotoxic intermediates from NP; further investigations will show whether this holds true for other



1-NITROPYRENE



3-NITROFLUORANTHENE

Fig. 1. Structures of 1-nitropyrene and 3-nitrofluoranthene

NO_2PAH, and what factors determine the relative importance of each route of metabolism. Thus we describe here a comparison of the activation pathways of NP and NFA (a NO_2PAH which has not yet been as well characterised as NP) in the Ames assay for reversion to histidine independence in Salmonella strain TA98 and in a forward mutation to 8-azaguanine resistance in Salmonella strain TM677 (Skopek et al., 1978, modified by Goto et al., 1985).

MATERIALS AND METHODS

Chemicals

1-Nitro[4,5,9,10-^{14}C]pyrene (^{14}C-NP; 10 mCi/mmole), unlabelled NP and NFA, and 3-nitro[3-^{14}C]fluoranthene (^{14}C-NFA; 13.8 mCi/mmole) were synthesised by Midwest Research Institute, Kansas City, MO. 3-Aminofluoranthene (AFA) was purchased from Aldrich Chemical Co., Milwaukee, WI, purified by recrystallisation, and acetylated to yield 3-acetylaminofluoranthene (AAFA). Other standards and reference compounds were obtained as described previously (Ball et al., 1984a, 1984b). Routine chemicals and solvents (reagent grade or better) were purchased from commercial suppliers, principally Sigma Chemical Co., St Louis, MO, and Burdick and Jackson Inc., Muskegon, MI.

Mutagenicity Assays

The Salmonella typhimurium plate-incorporation assay was carried out in strains TA98 (obtained from Dr. B. Ames, University of California, Berkeley, CA, USA), TA98NR and TA98/1,8-DNP_6 (from Dr. H.S. Rosenkranz, Case Western Reserve University, Cleveland, OH, USA) according to the procedures described by Ames et al. (1975), with the following modifications: minimal histidine was added to the base agar rather than to the soft agar overlay, and plates were counted at 72 rather than 48 hr. The forward mutation to 8-azaguanine resistance in Salmonella typhimurium strain TM677 (grown from a clone selected for maximum activity towards benzo(a)pyrene from a culture supplied by Dr. W.G. Thilly, Massachusetts Institute of Technology, Cambridge, MA) was performed scaled down ten-fold from the method of Skopek et al., 1978, as described by Goto et al., 1985. Chemicals were dissolved in DMSO for assay.

S9 Metabolic Activation System

The S9 fraction (9000 x g supernatant) used to provide exogenous metabolic activation was obtained from the livers of Aroclor-1254-treated male CD-1 rats (Charles River, Wilmington, MA, USA) as described by Ames et al., 1975, and stored frozen at -70°C until used. The NADPH-generating co-factor mix added to the S9 was made up as described by Ames et al., 1975.

Measurement of 1-Aminopyrene Formation

NP (20 µg/ml) was incubated with Salmonella typhimurium strain TA98, with S9 alone (final vol. 1 ml), and with TM677 in the presence and absence of S9 (final vol. 0.1 ml), under the conditions and concentrations utilised for the respective forward and reverse mutation assays (conducted under air, with no attempt at generating an anaerobic atmosphere). After 1 hr at 37°, an equal vol. of MeOH was added and mixed by vortexing. The incubation mixture was centrifuged (2000 rpm, 10 min) to precipitate protein, then the supernatant was sampled (20 µl) for high pressure liquid chromatography (HPLC) analysis. (Zorbax-ODS 4.6 x 250 mm column, Dupont Instrument Co., Wilmington, DE, eluted at 0.8 ml/min with a gradient of 80 to 100% MeOH in H_2O over 15 min). The 1-aminopyrene (AP) content of the supernatant was determined by fluorescence spectroscopy at 360 nm excitation, 430 nm emission (MPF-2A fluorometer, Perkin-Elmer Corp., Norwalk, CT.) The area of the peak

eluting with the retention time of authentic AP was measured electronically (Vista 4000 Data System, Varian Instrument Co., Palo Alto, CA) and calibrated by comparison with a standard curve constructed by injection of authentic AP. NP was quantitated analogously, by measurement of its UV absorbance at 280 nm.

Incubation of 3-Nitro[3-^{14}C]fluoranthene with S9

^{14}C-NFA (50 μM) dissolved in DMSO was added to Aroclor-treated rat liver S9 fraction (1 mg/ml in 0.1M HEPES buffer, pH 7.4) prepared as described (Ames et al., 1975). The reaction was started by addition of NADPH (1mM) and terminated (after 1 hr at 37°C) by extraction as described below.

Analysis and Quantitation of 3-Nitrofluoranthene Metabolites

The incubation mixture was extracted with ethyl acetate/acetone (2:1 v/v; 3 x 1 vol). The organic extract was concentrated by rotary evaporation, taken to dryness under a stream of N_2 gas, redissolved in MeOH (0.5 ml), then injected into an HPLC system consisting of a Varian Model 5050 HPLC (Varian Instrument Co., Palo Alto, CA) equipped with a Zorbax-ODS 4.6 x 250 mm column (Dupont Instrument Co., Wilmington, DE). The column was eluted at 1 ml/min with a linear gradient of 70% MeOH in H_2O rising to 100% MeOH over 20 min. The eluate was monitored for UV absorbance at 254 nm (UV-100 detector, Varian Instrument Co., Palo Alto, CA) and for fluorescence at 360 nm excitation, 430 nm emission (MPF-2A fluorometer, Perkin-Elmer Corp., Norwalk, CT). The eluate was collected in 30-sec fractions (Aliquogel B-200, Gilson Medical Electronics, Middleton, WI); the ^{14}C content of the fractions was determined by liquid scintillation counting (Model 2660 liquid scintillation counter, Packard Instrument Co., Downers Grove, IL).

RESULTS

The mutagenicity of NFA was characterized in the Ames histidine reversion assay and in the forward mutation assay, and compared to that of NP in the same two systems. Results are shown in Table 1. (Data on the activity of NP in the Ames assay are from Ball et al., 1984a). Since NP and NFA are isomers (M. Wt. 247), comparisons can equally well be made on the basis of direct weight or of molecular weight. Both compounds exhibited the same general pattern of responses; both were more active in the absence of S9 in the reversion assay, but required the presence of S9 to induce significant mutagenicity in the forward assay. Within each assay, NFA gave maximal responses at doses approximately one-tenth of those required for NP. Since mutagenicity of NFA in the Ames assay thus appeared to follow the nitroreductase-requiring pattern previously observed for NP, the activity of its reduced derivatives and potential metabolites was also examined in this system (Table 2). Direct-acting mutagenicity was lower with the amino derivative of NFA than with the parent compound, and was quite abolished by N-acetylation; a similar pattern had previously been shown for NP (Ball et al., 1984a). The S9-dependent activity of NP was also decreased by reduction, but further acetylation then increased activity beyond that of the parent compound; 6-hydroxy-N-acetylaminopyrene (a metabolite formed *in vivo*) was found to have ten times the S9-dependent activity of NP (Ball et al., 1984b) and may therefore be implicated in activation of NP in the whole animal. In contrast, both 3-aminofluoranthene and N-acetyl-3-aminofluoranthene were less active with S9 than was the parent compound; the activity of any further ring-hydroxylated derivatives remains to be ascertained.

The results obtained in the forward mutation assay with *Salmonella typhimurium* strain TM677 (activity of NP and NFA almost entirely S9-dependent) were in contrast to the general pattern seen in other Salmonella strains

Table 1. Comparison of the Mutagenicity of 1-Nitropyrene and 3-Nitrofluoranthene in Forward and Reverse Bacterial Mutagenicity Assays

	Histidine$^+$ Reversion (Ames et al., 1975) in Salmonella Typhimurium strain TA98[a]			8-Azaguanine Resistance (Skopek et al., 1978; Goto et al., 1985) in Salmonella TM677[b]		
Compound	Dose	- S9	+ S9	Dose	- S9	+ S9
Vehicle (DMSO)		20-30	40-50		1-2	2-4
1-Nitropyrene	0.1	152	71[c]	12.5	0.9	32
	0.5	771	144	50	1.2	54
	1.0	1475	246	100	2.0	40
3-Nitrofluoranthene	0.1	1670	118	1.25	0.8	6
	0.5	3265	460	2.5	1.6	41
	1.0	3055	1067	5	1.6	88
				10	3.8	94

[a] Dose is given as µg/plate, and results are expressed as His$^+$ revertants per plate.
[b] Dose is given as µg/ml, and results are expressed as 8-Azaguanine resistant mutants per 10^5 survivors.
[c] Data on NP in the Ames assay from Ball et al., 1984a.

in the Ames assay, where the presence of S9 reduces the activity of these NO_2PAH. Examination of the formation of AP from NP in strain TM677 under the exact conditions of the 1 hr incubation involved in the forward mutation assay indicated that this strain did not appear to metabolise NP at all, and only when S9 was included in the incubation mixture was AP formed in detectable amounts (Table 3). In contrast, both Salmonella TA98 and S9 alone (incubated under air, in concentrations corresponding to those used for the Ames assay) consumed at least 20 and 50% of the NP substrate respectively and produced measurable quantities of AP in 1 hr.

Table 2. Comparison of the Mutagenicity of Reduced Metabolites and Derivatives of 1-Nitropyrene and 3-Nitrofluoranthene in the Ames Histidine$^+$ Reversion Assay

	1-R-Pyrene[a]		3-R-Fluoranthene	
Substituent (R)	- S9	+ S9	- S9	+ S9
Nitro-	339[b]	60	2420	222
Amino-	93	33	43	113
N-Acetylamino-	≈ 0	414	≈ 0	61
6-Hydroxy-N-acetylamino	≈ 0	600	?	?

[a] Data from Ball et al., 1984a, 1984b.
[b] Mutagenicity values are given as revertants per nmole, calculated from the linear portion of the dose-response curve.

Table 3. Metabolism of 1-Nitropyrene to 1-Aminopyrene by Salmonella Strains TA98 and TM677 and by Aroclor-treated rat liver S9

Strain/Tissue	Percent Disappearance of substrate (1-Nitropyrene)	Formation of 1-Aminopyrene (pmoles/ml)
Salmonella TM677	< 5[b]	< 9[b]
Salmonella TM677 + S9	≈ 50	200
Salmonella TA98	20-30	51
Aroclor-treated Rat Liver S9	50-80	59

[a] Incubations were conducted under air for 1 hr at 37°C. Concentrations of organisms and/or tissue were as used in the corresponding mutagenicity assay. The substrate was 20 μg/ml of 1-nitropyrene (80 μM) throughout, corresponding to dose levels which produced a definite positive response in the forward mutation assay, but little signs of toxicity. The reaction mixture was quenched and extracted with an equal volume of MeOH, centrifuged to precipitate protein, then analysed by HPLC. 1-Aminopyrene was detected and quantitated by fluorescence spectrometry (Ex. 360 nm, Em. 430 nm), 1-nitropyrene by its UV absorbance at 280 nm.

[b] These values represent the limits of detection of the assay.

Rat liver S9 has been shown to metabolise NP to phenols, a dihydrodiol, AP and N-acetyl-1-aminopyrene (El-Bayoumy and Hecht, 1983; Ball et al., 1984a). ^{14}C-NFA was incubated with Aroclor-treated rat liver S9 in order to investigate the nature and extent of the metabolic transformations undergone by this NO_2PAH. After 1 hr at 37°C, 70-80% of the ^{14}C was extractable into ethyl acetate/acetone. Figure 2 shows the profile obtained by HPLC examination of the organic-extractable metabolites formed. Over 75% of the chromatographed ^{14}C eluted as unchanged NFA. Less than 1% of the ^{14}C eluted with the retention times of reference 3-aminofluoranthene and 3-acetylaminofluoranthene. This contrasts with previous findings for NP, which indicated that reduction and acetylation represented 17 % of the metabolism (over 5% of total ^{14}C) under air (Ball et al., 1984a). The predominant metabolite fraction (R_T 17-18 min) represented ≈ 15% of the organic-extractable ^{14}C. Other minor peaks amounted to 0.5-2% of the extractable radioactivity. Thus, including non-extractable material, a total of 40-45% of the NFA underwent transformation, which would correspond to ≈ 20 nmoles of substrate consumed per mg of S9 protein in 1 hr.

The peak eluting between 17 and 18 min was collected for further analysis. On rechromatographing this material resolved into one major and several minor peaks. The UV spectrum of the principal component was shifted to longer wavelengths in alkaline solution, indicative of a phenol; the 250 MHz proton n.m.r. spectrum showed the presence of a single component with 8 aromatic protons, with resonances shifted upfield from the corresponding peaks in the n.m.r. spectrum of NFA itself (results not shown). Assignment of the individual resonance peaks (confirmed by examination of the coupling constants and comparison with the spectrum of NFA) indicated the presence of a hydroxyl group, and excluded all positions of substitution other than carbons 8 or 9. Consideration of the reactivity of fluoranthene substituted on carbon 3 with a meta-directing group such as NO_2 towards

electrophilic substitution reactions (Campbell and Keir, 1955) would suggest that the latter position (carbon 9) would be more favoured. We therefore conclude that the principal metabolite fraction formed from NFA by Aroclor-treated rat liver S9 is 9-hydroxy-NFA.

The other minor metabolites eluting between 5 and 10 min may represent dihydrodiols, since on treatment with 1M HCl their retention times shift to 15-20 min, but this identification is not definitive.

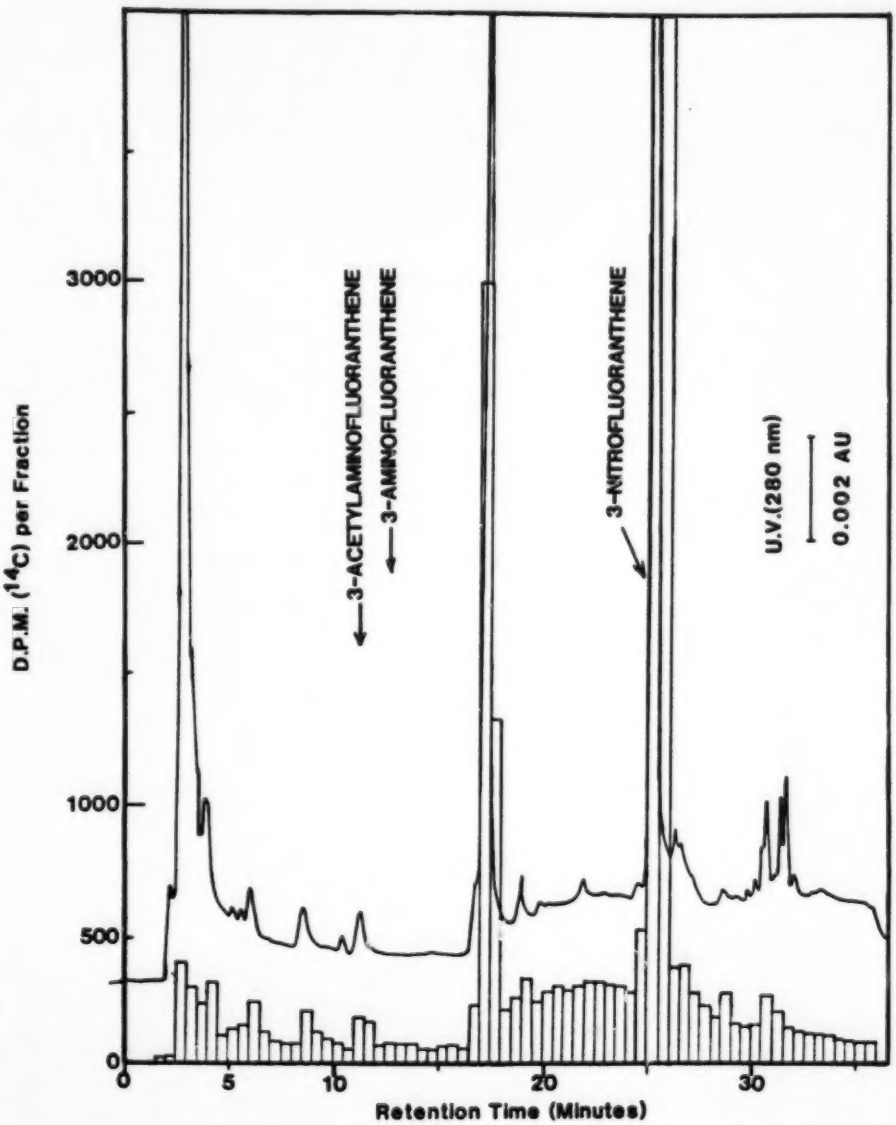


Fig. 2. HPLC Trace of Metabolites of 3-Nitrofluoranthene formed by Aroclor-treated rat liver S9 fraction. 3-Nitro[^{14}C]fluoranthene was incubated with 1 ml/ml protein and 1 mM NADPH for 1 hr at 37°C. Metabolites were extracted with ethyl acetate/acetone, concentrated then analysed by reverse phase HPLC and liquid scintillation counting.

In the Ames Assay NP and NFA both exhibit their maximal response in the absence of S9. The direct product of reduction (i.e. the amino derivative) is less mutagenic than the parent compound, confirming earlier conclusions that the active genotoxic intermediates involved are formed predominantly during the process of reduction of the nitro group. Unlike NP, acetylation of the aminofluoranthene does not lead to enhanced S9-dependent mutagenic activity. This suggests that activation in mammalian tissues through formation of the acetamido derivative, and generation of an electrophilic arylnitrenium ion by further metabolism along the pathways defined for carcinogenic aromatic amines such as 2-aminofluorene (Miller and Miller, 1981), may not be a mechanism applicable to all NO_2PAH.

In the forward mutation assay the presence of S9 is required for both NP and NFA to increase the frequency of mutation above control levels. This is in sharp contrast to the situation in the standard Ames assay, where the presence of S9 generally results in a decrease in the level of activity of most NO_2PAH. If nitroreductase activity (as measured by AP formation) is indeed required for generation of active intermediates from NO_2PAH, low reductase activity in Salmonella TM677 without S9, and enhanced activity in this strain in the presence of S9, might explain the requirement for S9 for expression of the mutagenicity of NP and NFA in the forward mutation assay.

The absence of detecteable levels of AP formation in TM677 suggests that this strain may lack the "classical" bacterial oxygen-insensitive nitroreductase which is the primary enzyme involved in activation of NP (Mermelstein et al., 1981). The Salmonella strain from which TM677 is derived is TA1535, which is fully competent with respect to nitroreduction (Messier et al., 1981). A substantial loss of enzymic activity would therefore have to have taken place in the process of formulating the new strain. If indeed TM677 is deficient in oxygen-insensitive nitroreductase, then the presence of active S9 fraction in the incubation mix might in itself act as an oxygen scavenger, to allow expression of a bacterial oxygen-sensitive nitroreductase. This possibility is supported by the fact that AP production is higher in TM677 + S9 than in S9 alone. Alternatively, oxidative metabolism by rat liver S9 fraction can form products which themselves exhibit mutagenic activity. This was shown to be the case for NP (El-Bayoumy and Hecht, 1983; Ball et al., 1984a); the genotoxic characteristics of the principal metabolite of NFA formed by rat liver S9, here identified as 9-hydroxy-NFA, remain to be evaluated in this respect.

Therefore both enhanced nitroreduction in the presence of S9 and S9-specific oxidative metabolism may contribute to the activation of the NO_2PAHs NP and NFA in the forward mutation assay with Salmonella typhimurium strain TM677.

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